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Mineralized soft-tissue structure and chemistry in a mummified hadrosaur from the Hell Creek Formation, North Dakota (USA)

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An extremely well-preserved dinosaur (*Cf. Edmontosaurus* sp.) found in the Hell Creek Formation (Upper Cretaceous, North Dakota) retains soft-tissue replacement structures and associated organic compounds. Mineral cements precipitated in the skin apparently follow original cell boundaries, partially preserving epidermis microstructure. Infrared and electron microprobe images of ossified tendon clearly show preserved mineral zonation, with silica and trapped carbon dioxide forming thin linings on Haversian canals within apatite. Furthermore, Fourier transform infrared spectroscopy (FTIR) of materials recovered from the skin and terminal ungual phalanx suggests the presence of compounds containing amide groups. Amino acid composition analyses of the mineralized skin envelope clearly differ from the surrounding matrix; however, intact proteins could not be obtained using protein mass spectrometry. The presence of endogenously derived organics from the skin was further demonstrated by pyrolysis gas chromatography mass spectrometry (Py-GCMS), indicating survival and presence of macromolecules that were in part aliphatic (see the electronic supplementary material).

Keywords: hadrosaur; Hell Creek; soft tissue

1. INTRODUCTION

The recognition of dinosaur soft-tissue structures and organic molecules preserved inside bone has been previously reported in a number of publications (see Schweitzer *et al.* 2007 for review). The presence of both organic structures and molecules in dinosaur soft tissues such as skin, terminal ungual phalanx sheath, tendon or degraded collagen fibres has been only rarely reported (Schweitzer *et al.* 1999a,b; Lingham-Soliar *et al.* 2007). Here we report the preservation of soft-tissue replacement structures and the presence of organic molecules associated with a hadrosaur dinosaur, *Edmontosaurus* sp. (MRF-03), from the Upper Cretaceous, Hell Creek

Formation of North Dakota, USA. Large areas of uncollapsed skin ‘envelope’ are preserved through early mineralization around much of the fossil including the tail, legs and an arm. Skin impressions observed on most other dinosaur ‘mummies’ are predominantly interpreted as trace fossils (Sternberg 1953; Martill 1991; Kellner 1996; Murphy *et al.* 2002); however the integument of MRF-03 displays both depth and structure (figures 1 and 2).

The depositional environment aided the rapid precipitation of minerals around and within the skin replacing the hide. Similar mineralization of dinosaur skin has been noted from the lacustrine Las Hoya limestone (Briggs *et al.* 1997) and a marine carbonate mudstone (Martill *et al.* 2000). Structures consistent with melanosomes have also been imaged in fossil feathers from the marine Crato Formation (Early Cretaceous) of Brazil (Vinther *et al.* 2008), although previous studies

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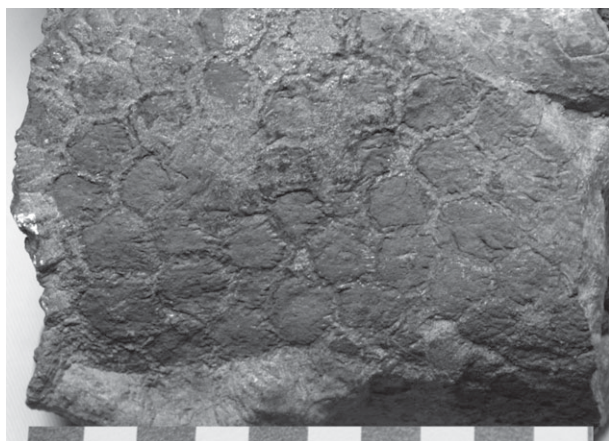


Figure 1. Dorsal surface of mid-section from the forearm of MRF-03. Detail of the fossil displaying three-dimensional aspect of skin preservation. The skin is not a typical trace fossil, but mineralized integument with depth and structure. Scale bar, 10 cm.

suggested that similar features were microbial in origin (Wuttke 1983; Bingham *et al.* 2008). In comparison, MRF-03 is preserved in a terrestrial, probably waterlogged, setting. There has also been mineral replacement of the epidermal laminae of the keratinous sheaths on the terminal ungual phalanx of several digits, indicating that mineralization outpaced the decomposition of these structures. Structural biomaterials present would be expected to fall within what is predicted for the extant phylogenetic bracket (EPB) of crocodilia and Aves (Witmer 1995), constraining the beta-keratin composition for any dinosaur 'keratinous' structure analysed (Manning *et al.* 2006).

The remains of hadrosaur MRF-03 were preserved through rapid burial on the margins of a sandy river channel. Sediment enclosing the specimen is mainly composed of fine sand-sized grains of quartz, feldspars and rock fragments with some higher plant-derived organic matter (Johnson 2002; Fricke & Pearson 2008). Isotopic and chemical analysis of sedimentary cement gives insight into the environment of preservation. Stable isotopic compositions of carbonate cements (CaCO_3 and FeCO_3 , identified by optical microscopy, electron microprobe and SEM; $\delta^{13}\text{C}$ range 1.58–7.08 and $\delta^{18}\text{O}$ range –5.13 to –6.67 both per mil relative to pee dee belemnite (PDB)) in concretions associated with MRF-03 suggest that the pore waters were purely meteoric in origin (with no effects of evaporation) and that methanogenesis in an anoxic environment contributed significant bicarbonate. Intensely reducing porewaters, generated by the decay of plant material, caused oxidized iron and manganese species to be reduced and the feldspars and rock fragments to partially dissolve. Calcium, reduced iron and reduced manganese in solution were then available to replace the soft tissue with carbonate minerals. Crucially, supply of iron to the rotting dinosaur was maintained by Fe-rich groundwaters that preferentially flowed through the higher porosity sandy units that were deposited as channel sands rather than through the mud-dominated over-bank deposits. Rapid precipitation of carbonates was therefore critical in this process.

2. METHODS

The soft-tissue structures from MRF-03 were analysed with a range of techniques to elucidate their structure and composition. First, imaging of selected areas was completed using both electron microscopy to study the mineralization process and scanning FTIR analysis in order to screen the sample for the potential presence of organic molecules. Next, directed by the imaging results, we applied a range of state-of-the-art biological and geochemical analytical techniques to targeted samples in an attempt to identify any organic molecules associated with the specimen, including amino acid analysis, polyacrylamide gel electrophoresis (PAGE) and mass spectrometry. Organic analyses were also completed on sediment blanks taken from the same lithology as controls.

The specimen MRF-03 of the Marmarth Research Foundation is tentatively assigned to approximately *Edmontosaurus* sp., based on the osteology of the pelvic and pectoral regions (Brett-Surman & Wagner 2007). However, this will be confirmed when preparation of the specimen is completed. We sampled a mineralized organic sheath structure surrounding part of a terminal ungual phalanx of a pedal digit, skin from the base of the tail and an ossified tendon from the neural spine of the proximal caudal series. Each of the samples displayed discrete structural and chemical information. Below we present the results for each of the three tissue types.

3. RESULTS

(a) Skin structure and chemistry

The preserved skin thickness varies across the body of MRF-03 (figure 2a), but averages 2.5–3.5 mm in depth in the caudal region where a natural break between the body and tail allowed access to the integument in cross section. An organic-rich band on the upper and lower surfaces of the skin in polished thin section constrains the depth of the structure (figure 2b). The skin has been replaced by carbonate mineralization that is both chemically and texturally different from the surrounding sedimentary matrix. Equant cell-like structures within the skin are evident in thin section and range between 5 and 20 μm across (figure 2c).

In order to further map the fine structure of the skin, Environmental Scanning Electron Microscope (ESEM) images of an uncoated thin section of skin from MRF-03 were obtained. The upper portion of the skin in one thin-sectioned sample clearly showed parallel structures that might be expected for the *stratum corneum* of a vertebrate skin section (figure 2d), comparable with extant vertebrates (Matoltsy 1986).

An electron microprobe was used to map the chemistry of the sectioned skin of MRF-03. The calcium abundance map (figure 3) clearly shows a cell-like texture within and constrained by the skin, revealing two distinct regions. The interior (lower) region (1.5–2.5 mm thick) has low calcium content, compared with the exterior (upper) surface (approx. 1 mm thick). We interpret this as early carbonate growth preserving the original tissue texture of the dinosaur skin. Precipitate texture (approx. 20–30 μm lateral width) and overall cross-sectional thickness of the postulated 'skin' are comparable to the cell texture and skin thickness of extant organisms (Bada *et al.* 1973). Cathodoluminescence imaging of a similar region, completed independently at the University of Liverpool, shows similar structures (figure S1, electronic

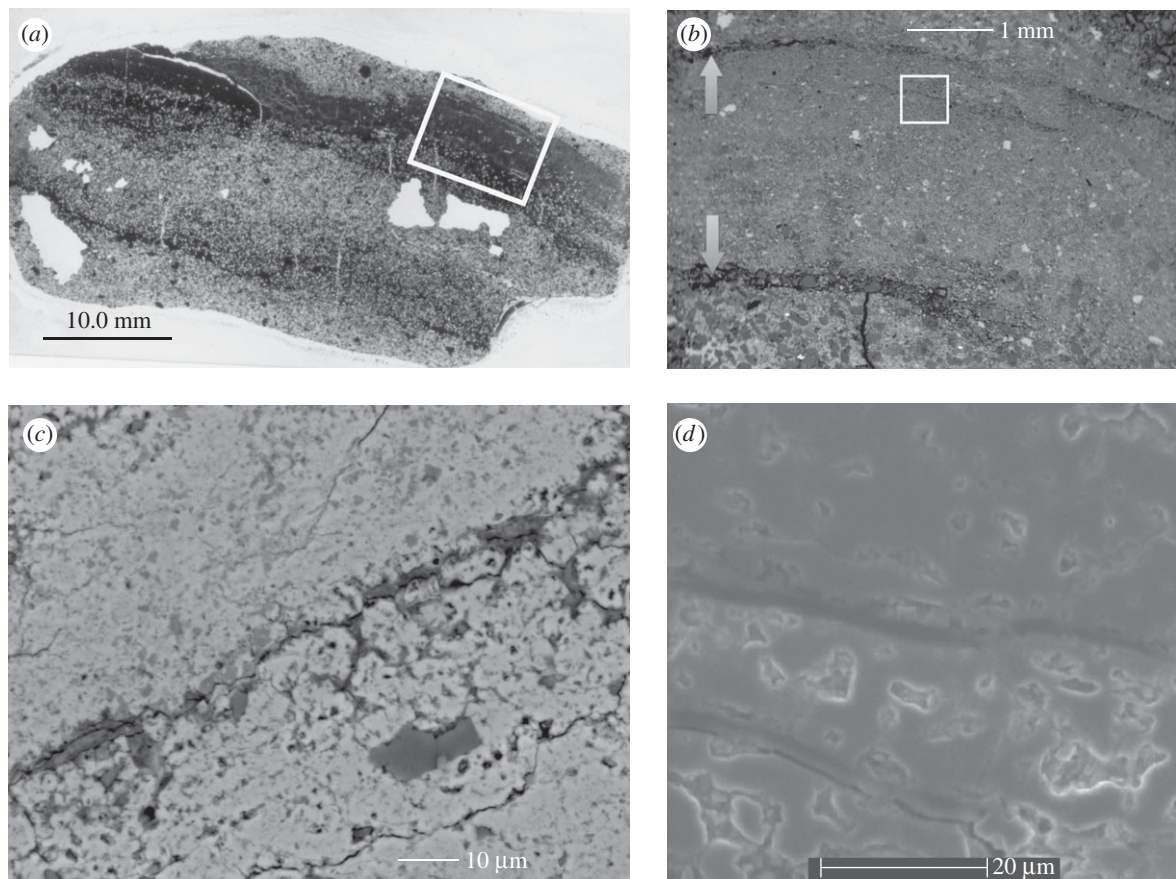


Figure 2. (a) Transmitted light micrograph image of skin: black/grey layer in the upper portion of the slide is skin. White box indicates area scanned in (b). (b) Skin in thin section imaged via back-scattered electron imaging (BSEI). Arrows indicate the dark organic-rich layers that bound the skin at top and bottom. Small rectangle indicates the approximate location of area detailed in (c). (c) BSEI image of the skin thin section showing structures that closely resemble cellular aggregates both in size and in morphology. Epidermal boundary between distinct tissue types (d). ESEM image taken from a non-carbon-coated cross section of skin. Image indicates laminated structure within the upper laminated portion of the skin from MRF-03.

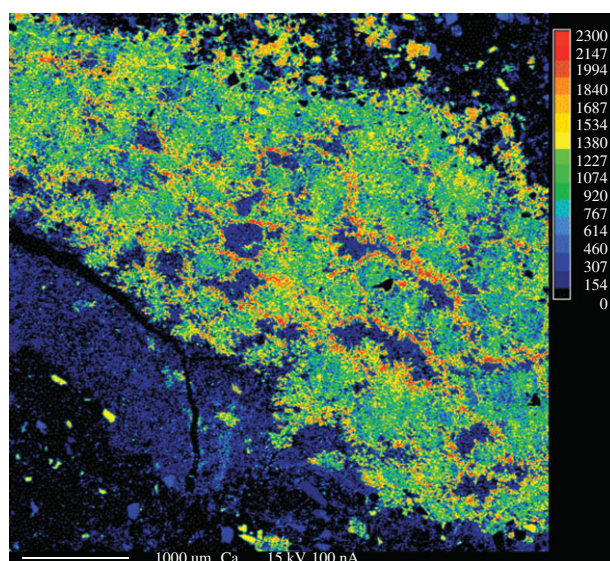


Figure 3. Electron microprobe false colour intensity map of calcium distribution within a thin section of the skin of MRF-03 (map size: $450\ \mu\text{m} \times 365\ \mu\text{m}$). Calcite (identified by SEM and optical microscopy) has precipitated as either hollow rings or small equant crystals, approximately $20\ \mu\text{m}$ in diameter. Total inferred epidermal thickness here is 2.5–3.5 mm.

supplementary material). Incomplete preservation is possible; however, these observations constrain the minimum thickness of the remaining epidermis for MRF-03.

(b) FTIR analysis of the terminal ungual phalanx

An iron carbonate matrix that persisted on the distal toes of each pes was reminiscent of organic, sheath-like structures that would have enclosed the bone of each terminal ungual phalanx. The composition of such structures in extant vertebrates is conservative and typically composed of keratin. There are two main types of keratin: alpha and beta. The EPB of dinosaurs (Witmer 1995) suggests beta-keratin would be the structural form produced by this group of animals. Given that beta-keratin is a relatively robust structural protein, we initially focused our organic analysis here. The surface of the iron-rich carbonate that formed the matrix for this structure showed clear signs of breakdown, indicating that the original surface of the terminal ungual phalanx sheath was not present and that a degraded subsurface layer within the keratinous sheath remained.

To avoid contamination, grains (less than 1 mm) were removed from the terminal ungual phalanx sheath matrix and analysed with no further preparation. Stingray FTIR

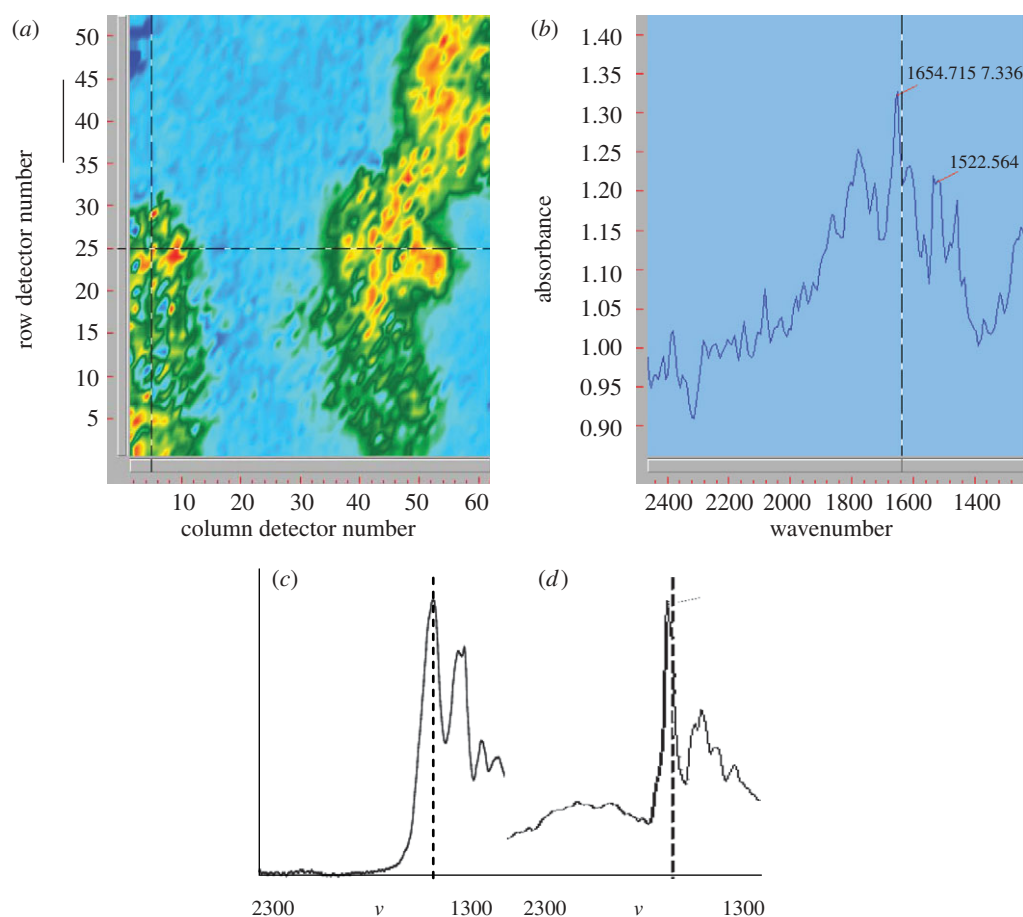


Figure 4. Stingray FTIR (transmission mode) map (a) of the intensity of absorption at 1654 cm^{-1} corresponding to a probable distribution of amide (I) groups on sediment grains sampled from the sheath covering the terminal ungual phalanx region of MRF-03. The corresponding spectrum (b, row, 25; column, 5) (from the point on the crosshairs in the map) suggests that both amide I and II groups are present in the mapped organic residue (corresponding peaks located at 1654 and 1522 cm^{-1} , respectively), and thus is inferred to indicate the presence of protein or protein breakdown products. Reference spectra from reptilian (c, terminal ungual phalanx from juvenile *Crocodylus porosus*) and avian (d, down feather from *Columba livia*) beta-keratin are shown for comparison; amide I peaks are indicated with vertical dashed lines at 1630 and 1644 cm^{-1} , respectively.

mapping showed an organic coating on many of the grains (figure 4) that contained an absorption band corresponding to the characteristic amide I band (approx. 1650 cm^{-1}). Mapped absorption at the amide II band position (approx. 1520 cm^{-1}) showed nearly identical zonation. This FTIR result was repeated on 13 samples from the structure coating the terminal ungual phalanx, 11 suggested the presence of remnant organic molecules. The position and appearance of the FTIR bands of the amide I and II groups present in the terminal ungual phalanx region are directly comparable to the beta-keratin samples taken from pigeon down and crocodile terminal ungual phalanx (figure 4).

(c) Amino acid analyses

Amino acid composition and racemization analyses were carried out as potential screening methods to identify samples suitable for protein analysis. The relative amino acid concentrations for the majority of samples collected from MRF-03, including fragments of the keratinous sheath and skin, were similar to those observed in the surrounding matrix and woody material, but at concentrations greater by nearly an order of magnitude, possibly indicative of microbial contamination

(figure 5a). The amino acid racemization results also indicated the presence of microbial contamination with higher *D/L* values (figure 5b) in slow racemizing amino acids (such as alanine) than in faster racemizing amino acids (such as aspartic acid and asparagines) (Bada *et al.* 1973). The presence of microbial biofilms may be misleading when attempting to determine the presence of endogenous organics (Kaye *et al.* 2008). However, the skin envelope sample taken from the base of the tail does exhibit a distinct composition potentially indicative of fibrous structural proteins such as collagens and keratins. Given these amino acid compositions, the skin envelope was then pursued as the region most likely to contain endogenous beta-keratin and was further analysed by various proteomics-based techniques.

(d) Proteomics analyses

A method that successfully separated out 12 kDa beta-keratin proteins from barnacle goose claw using a size exclusion and PAGE protocol was developed at the Wolfson Molecular Imaging Center (WMIC), but when applied to samples taken from the terminal ungual phalanx sheath and skin regions it was unsuccessful at recovering intact proteins. When the size exclusion

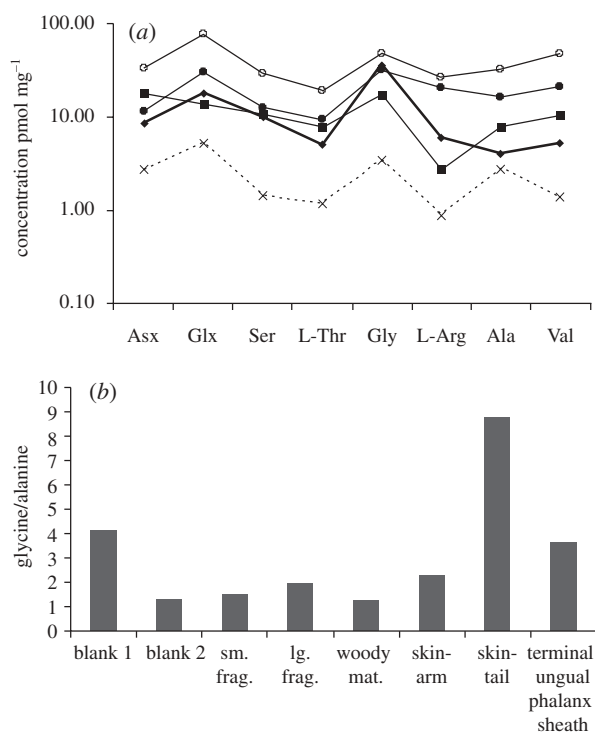


Figure 5. (a) Amino acid composition plots of four samples taken from the dinomummy and one of the sediment blanks. The skin envelope appears to contain a distinct composition, potentially containing endogenous protein. Asx, asparagine; Glx, glutamic acid; Ser, serine; L-Thr, L-threonine; Gly, glycine; L-Arg, L-arginine; Ala, alanine; Val, valine. Cross, sediment blank; small open circle, small fragment of keratinous sheath; small filled circle, large fragment of keratinous sheath; large filled square, skin isolated from arm; diamond, skin envelope from base of tail. (b) Glycine/alanine ratios in samples taken from various locations within the specimen. The skin in the tail region had the highest glycine/alanine ratios, similar to those expected in structural proteins such as collagens and keratins, and was therefore selected as the best candidate for proteomics analysis.

step was removed from the preparation protocol, low-molecular-weight fractions (less than 12 kDa) were clearly observed on the electrophoresis gel for samples from the sheath covering the terminal ungual phalanx region and from the skin, thus indicating the presence of organic material. *Low-molecular-weight fractions were not observed by this technique in any of the sediment controls taken near the fossil.*

Independent analyses at the University of Manchester School of Life Sciences using matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) and liquid chromatography-electrospray ionization (LC-ESI) following various protocols (see electronic supplementary material) were consistent with the results produced by the WMIC in that low-molecular-weight peaks at m/z 1100–2200 were observed. However, the identity of these could not be ascertained owing to the very low signal and poor quality MS/MS spectra obtained, clearly a fundamental issue when dealing with such ancient specimens. Some identical peaks were determined by this method in the sediment samples, which further indicates the necessity to obtain unambiguous MS/MS spectra before making claims to the identification of endogenous beta-keratin, despite the promising amino acid composition results.

(e) Pyrolysis of skin

However, the presence of organic compounds specifically associated with the skin envelope was further demonstrated by Py-GCMS (figure 6). The pyrolysates reveal a substantial difference in the aliphatic polymer from MRF-03 skin samples when compared with the associated sediment. Py-GCMS of the skin generated *n*-alkanes/*n*-alken-1-ene homologues ranging in carbon number from C₉ to C₃₆ with a trimodal distribution of *n*-alkanes (figure 6a; maxima C₁₁, C₁₅ and C₂₇). In comparison, the *n*-alkane/*n*-alken-1-ene homologue distribution pattern in the enclosing sediment differs considerably, ranging from C₉ to C₃₀ but dominated by the C₁₀–C₁₈ *n*-alkanes with a maximum at C₁₁ (figure 6b). The observed differences are inconsistent with an origin solely via migration from enclosing sediment and thus must have been derived endogenously. This suggests that the organics present in the skin envelope include a macromolecule that is in part aliphatic. Comparable to earlier studies on plant and insect fossils, these aliphatic components are interpreted to be the result of a process of *in situ* polymerization of original organic compounds derived from the hadrosaur (Gupta & Pancost 2004; Gupta *et al.* 2006, 2007a,b).

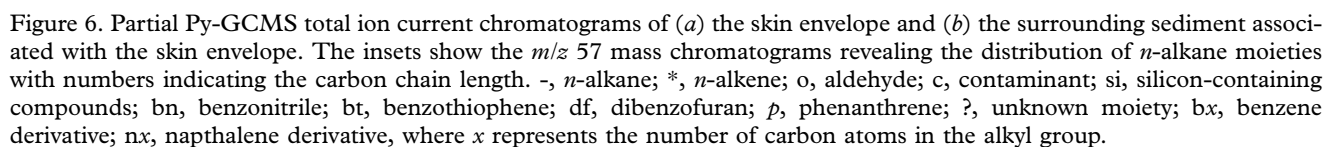
(f) X-ray diffraction of terminal ungual phalanx

XRD of samples taken from the terminal ungual phalanx sheath region was also completed, but this did not show distinct diffraction peaks corresponding to beta-keratin, corroborating the conclusion from the proteomic analysis that any original beta-keratin is probably severely degraded or extremely dilute. However, a broad background was observed, consistent with the presence of a large percentage of organic matter being contained in this region (along with quartz and feldspar). In contrast, XRD of sediment samples taken from in and around MRF-03 did not show such a background, consistent with the fact that Py-GCMS showed that the macromolecular composition of the sediment was significantly different from that observed in the skin (figure 6).

(g) Combined analyses of tendon

FTIR, XRD and electron microprobe analyses were also conducted upon a polished thin section of tendon (transversospinalis) (Organ 2006) recovered from a neural spine in the caudal region of MRF-03. XRD showed that the tendon is dominantly composed of the ossifying mineral apatite (bio-apatite: $\text{Ca}_{10-y+\gamma}\text{Na}_y(\text{PO}_4)_{6-x}(\text{CO}_3)_x(\text{OH})_{2-x+\gamma}$; Skinner 2000), and peaks corresponding to quartz or other pure SiO_2 phases were not present. Electron microprobe maps and point analyses showed that thin (5–25 μm thick) concentric coatings of pure SiO_2 were present on remnant Haversian canal structures (figure S2, electronic supplementary material). Because these coatings did not produce diffraction peaks, we assume that they are amorphous. Apatite is deposited when the organism was still alive as part of normal growth processes, as observed in extant vertebrates (Abdalla 1979). Amorphous silica most probably precipitated *post-mortem*, supplied from solutes in geochemical fluids.

FTIR maps, as shown in figure 7a (mapped at approx. 1170 cm^{-1}), also clearly show the presence of Haversian



A longitudinal section of the ossified epaxial tendon (figure S3, electronic supplementary material) shows that the incorporation of carbon dioxide is indeed associated with the Haversian canals, but is not uniform along the length of the structure, rather it is discontinuously spread over the surface of the canal. Concentric zonation in cross section of another Haversian canal is shown strikingly via a total reflectance infrared map (figure S4,

4. CONCLUSIONS

Mineralized soft-tissue structures preserved in the skin envelope, terminal ungual phalanx sheath and tendon resemble those observed in extant sections of analogous avian skin and tendon (Abdalla 1979; Weir & Lunam 2004; Adams & Organ 2005). The skin of extant vertebrates is commonly composed of two different tissues that are closely apposed to each other: a surface epidermis (constructed from multiple layers of epithelial cells) and an underlying dermis composed of dense connective tissue. Skin-like structures observed in MRF-03 suggest that the epidermis has been partially preserved in the area sectioned. Cell-like structures observed via BSEI and microprobe imaging (figures 2c and 3, respectively) support the potential presence of an epidermal layer comparable to extant vertebrates. The size of the cell-like structures (approx. 5–30 μm) is within the size range expected for skin cells (Fusenig 1986; Litzgus *et al.* 2004). The imaged partial epidermal

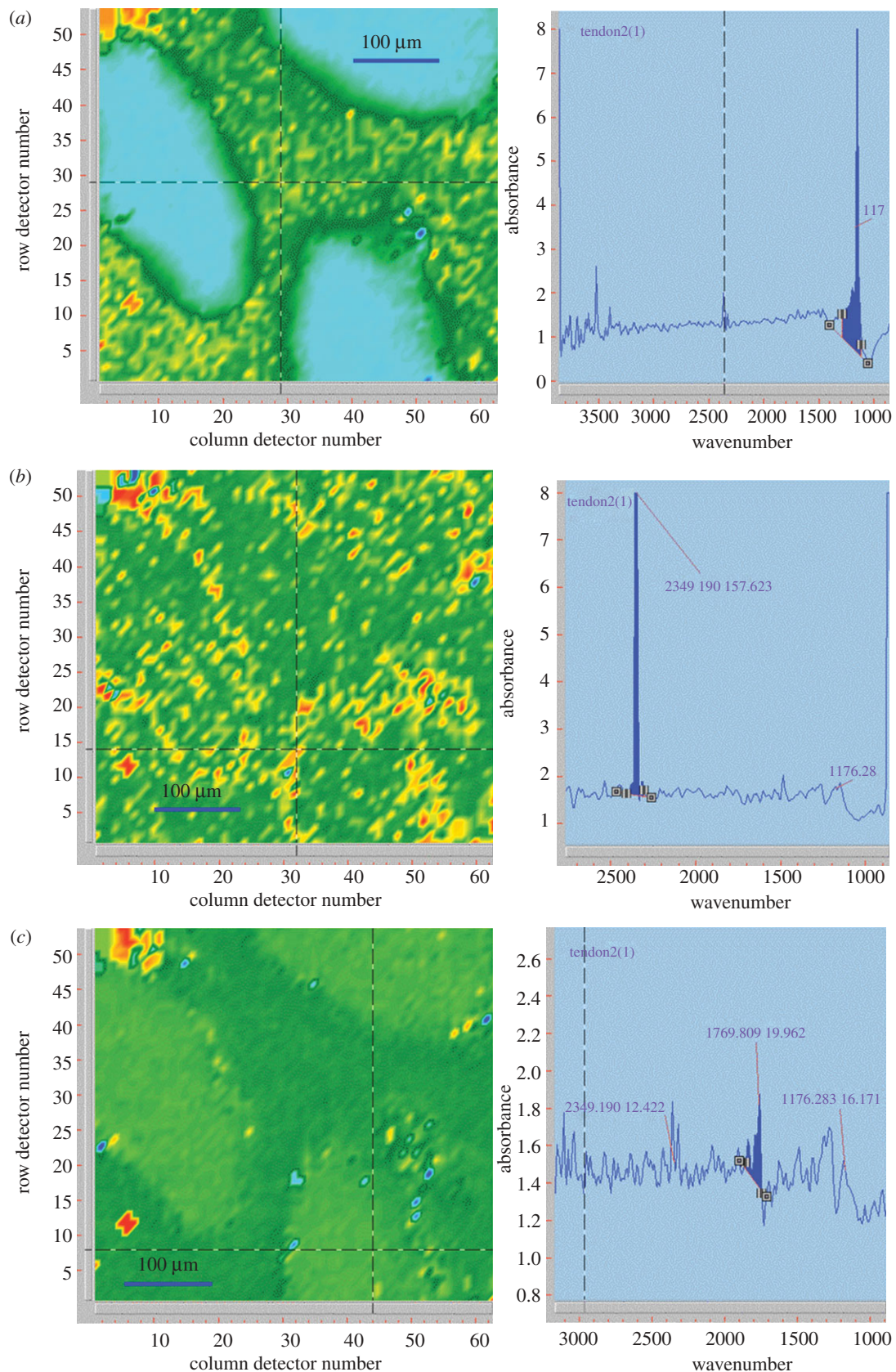


Figure 7. Stingray FTIR (reflection mode) images (left) and spectra (right) of a sample taken from a section of ossified epaxial tendon from MRF-03. This shows a cross section through three Haversian canals. (a) Map of the intensity of adsorption at 1170 cm^{-1} —crosshairs on the map (left) show from where the spectrum (right) has been selected. The Haversian canals within the apatite are clear. (b) An approximately $20\text{ }\mu\text{m}$ thick carbon dioxide-rich layer on the apatite is evident, again crosshairs on the map show where the spectrum (right) originates from. (c) Absorption bands at approximately 1770 cm^{-1} noted at several places on the sample mapping them showed clear structural control and suggests that organic material may persist associated with the canals.

thickness (3.5 mm) sets a minimum constraint for this organism and is comparable to that of several vertebrates such as humans (0.8–1.5 mm) (Matoltsy 1986), rhinoceros (15–25 mm) (Shadwick *et al.* 1992), hippopotamus (15–20 mm) (Shadwick *et al.* 1992) and elephant (10–15 mm) (Harkness & Harkness 1965).

Chemical mapping and amino acid analyses of MRF-03 clearly indicate that the composition of the preserved specimen differs from that of the surrounding sediment and maintains structures strongly reminiscent of soft tissue. Rapid burial, combined with methanogenesis in a depleted oxygen environment, contributed significant bicarbonate to the system. Intensely reducing porewaters, generated by the decay of plant material, caused oxidized iron species to be reduced and feldspar and rock fragments to partially dissolve. The reduced iron in solution rapidly replaced the soft tissue with carbonate minerals with dissolved silica lining the Haversian canals of the tendon. Mineralization apparently outpaced microbial decay processes, thus ensuring high-fidelity preservation of some integument structures. This rapid mineralization also ensured that some breakdown products of organic molecules at the point of burial, whether endogenous to MRF-03 or of microbial origin, were preserved within the mineral matrix.

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P.M.M. did FTIR analysis and interpretation; A.M. provided MALDI analytical assistance; E.J. carried out size separation, PAGE and MALDI-MS; A.G. completed MALDI-MS analyses; M.B. and O.R. carried out further MALDI-MS and LC-ESI-MS analyses; J.H.S.M. did optical and BSEI imaging; J.M. completed CL imaging and isotopic analysis; T.L. found the specimen and contributed material; P.L.M. performed ESEM and EMP and assisted B.E.v.D. with Py-GCMS analyses, managed the analytical programme and wrote the manuscript; R.A.W. planned the EMA, FTIR and XRD analyses, analysed the data and co-wrote the manuscript. All authors discussed and commented on the manuscript.

The authors declare no competing financial interests.

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